Evidence for Non-DLVO Hydration Interactions in Solutions of the Protein Apoferritin

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We have studied molecular interactions in solutions of the protein apoferritin by static and dynamic light scattering. When plotted against the electrolyte concentration, the second osmotic virial coefficient exhibits a minimum. The ascending branch of this dependence is a manifestation of a surprisingly strong repulsion between the molecules at electrolyte concentrations about and above $0.2M$, where electrostatic interactions are suppressed. We argue that the repulsion is due to the water structuring, enhanced by the accumulation of hydrophilic counterions around the apoferritin molecules, giving rise to so-called hydration forces.

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The interactions between the solute molecules in protein solutions affect their respective biological functions and determine the stability of the solutions with respect to aggregation, liquefaction, and other phase transformations. Furthermore, the pathways of protein crystal formation (protein crystals are the main source of structural information about the protein molecules) are largely defined by the forces acting between the molecules.

Solutions of globular proteins in many aspects resemble colloidal suspensions: The solute species are charged Brownian objects with sizes between a few nanometers and a few tens of nanometers. Hence, the theoretical approaches developed for colloids have been applied to proteins. Typically, interactions and stability of lyophobic colloids and globular proteins are treated in terms of Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory. This model is based on the balance between van der Waals attraction and electrostatic repulsion [1,2]. While appropriate for a wide variety of systems, the DLVO approach failed to account for some experimentally observed features of stability behavior. Hence, other types of interactions have been put forth [3]. For instance, besides the charge repulsion [4], higher-order multipole terms, leading to attraction, could also be present [5,6].

In this Letter we present static and dynamic light scattering results on the interactions between molecules in solutions of apoferritin, the hollow shells of ferritin, an iron storage protein with nearly spherical shape and diameter of $\sim$13 nm. At high electrolyte concentrations we found surprisingly high virial coefficients and argue that they are a consequence of non-DLVO hydration repulsion between the molecules. These results highlight the role of small solution components in protein interactions: They are not merely a part of the background responsible for screening the electrostatic forces but may interact with the protein molecules to modify the associated solvent structures and alter the intermolecular interactions.

Prior to light scattering data collection, the protein was purified by gel filtration chromatography to remove dimers, trimers, and higher order aggregates present in the commercial material [7]. The obtained monomers were dissolved in a $0.2M$ sodium acetate (NaAc) buffer with $p\mathrm{H} = 5.0$ at which crystallization is typically carried out. Since the $p\mathrm{I}$ (isoelectric point) of this protein is about 4.0 [8], at the chosen $p\mathrm{H}$ the monomers are negatively charged.

Dynamic and static light scattering experiments were performed in parallel using a Brookhaven 200 SM goniometer and correlator equipped with a He-Ne laser (Spectra Physics, 127 V/35 mV), operating at a wavelength of 632.8 nm. The refractive index increments, needed for interpretation of the static light scattering data, were determined for all solutions used at the same wavelength by a Wyatt Technologies Optilab device. All light scattering measurements were carried out at 20 ± 0.1°C or 293 K.

Dynamic light scattering allows determinations of the hydrodynamic radius a of the solute species from the cumulant expansion of the autocorrelation function. We found that $a = 6.35$ nm and is independent of protein and precipitant concentrations. This value is in good agreement with crystallographic [9] and atomic force microscopy [10] determinations of the apoferritin monomer size, and we conclude that the studied solutions contain only this protein species.

Static light scattering allows determination of the molecular weight M_w and second osmotic virial coefficient A_2 of solutions, using the relationship between the Rayleigh ratio R_θ and the mass concentration of the protein, C [11]

$$KCM_w/R_\theta = 1 + 2A_2M_wC\,, \tag{1}$$

where

$$K = (2\pi n_0/\lambda^2)^2(dn/dC)^2/N_A\,. \tag{2}$$

In Eqs. (1) and (2) N_A is Avogadro's number, n_0 is the refractive index of the buffer solution, λ is the wavelength of the scattered light, and dn/dC is the refractive index increment with protein concentration. The second virial coefficient can be presented in dimensionless form

$$B_2 = 3A_2M_w^2/4\pi N_A a^3. \tag{3}$$

Equation (1) then becomes

$$KCM_w/R_\theta = 1 + 2B_2\phi\,, \tag{4}$$

where ϕ is the protein volume fraction. The presentation of the static light scattering results in terms of Eqs. (1) or (4) is often called a Debye plot. The value of the dimensionless second virial coefficient B_2 is an integral characteristic of the intermolecular interactions. If the interactions are limited to inaccessibility of the volume occupied by one particle to the others, "hard spheres potential," $B_2 = 4$ [12]. Values >4 indicate even stronger repulsion, while lower and negative values indicate attraction between the solute species.

The static light scattering results in terms of Debye plots at various concentrations of the buffer cations Na^+ are shown in Fig. 1. The data in Fig. 1(a) are for concentrations of Na^+ from $0.01M$ (upper series) to $0.15M$ (lower series), while those in Fig. 1(b) are for concentrations from $0.15M$ (lower series) to $0.25M$ (upper series). The molecular mass obtained using Eq. (1) is ~450 kD for all cases except the highest two buffer concentrations ($0.20M$ and $0.25M$) where it was somewhat higher ~475 kD. The slope of the curves initially decreases with the electrolyte concentration. Further increase of the electrolyte concentration, however, leads to an increase of the slope, after a minimum at about $0.15M$ NaAc. These two trends are more clearly visible in Fig. 2 where the second osmotic virial coefficient B_2 is plotted against the concentration of Na^+. The decrease in repulsive forces with higher electrolyte concentration in the range $0 < [Na^+] < 0.15M$ is attributable to a classical DLVO effect [1,2], enhanced screening of the electric charges on the surface of the protein molecules by the more concentrated electrolyte. However, the increased repulsion at $(0.20-0.25)M$ is not amenable to explanation within the framework of the DLVO theory [1,2].

We interpret the observed trends of the second virial coefficient variations with the salt concentration as follows. At low ionic strength the overall interaction is governed by electrostatic repulsion. It decreases with the addition of electrolyte because of charge screening. The most likely mechanism for increased repulsion at higher concentration of Na^+ involves accumulation of hydrated sodium cations (recall the negative charge on the apoferritin molecules) in the vicinity of the protein surface. This gives rise to repulsion, with the forces acting between the molecules being called hydration forces [3,13–17]. A similar minimum in the dependence of a typical stability factor (Wuchs' factor) on the concentration of NaCl has been observed in experiments on coagulation kinetics of protein-stabilized latexes and attributed to variations in the balance between electrostatic and hydration forces with salt concentration [18].

The origin of the hydration forces still lacks a unique identification and appears to be system specific. They have been attributed to water structuring in the vicinity of the interacting surfaces [14], or to local variation of the dielectric permittivity due to the hydrated ions in the double layer [15,16]. The water orientation effect has been questioned and entropy generated repulsion due to thermal mobility of surface groups has been suggested instead [17]. Other electrostatic theories of the hydration repulsion have also been put forth [19].

To test if the proposed combination of electrostatic and hydration forces can account for the observed variations of the B_2, below we use generally accepted forms of these two types of forces. We show that quantitative correspondence to the experimental observations results from parameter

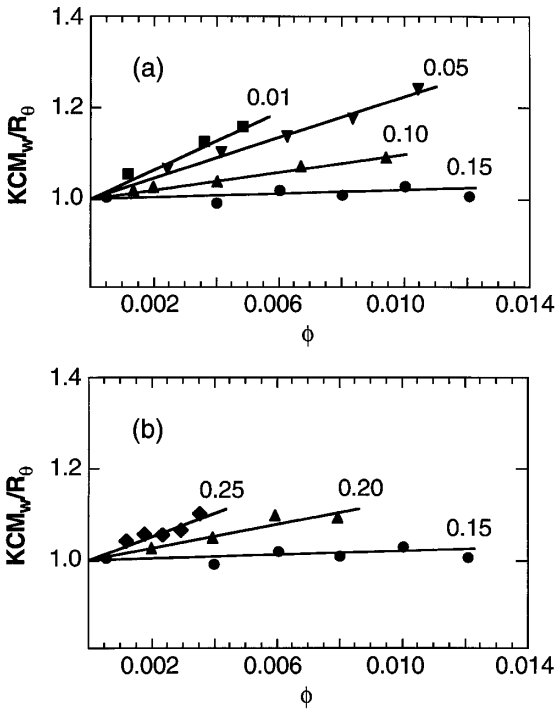

FIG. 1. Debye plots for apoferritin solutions in NaAc buffer. (a) Buffer concentrations in the range $(0.01-0.15)M$. (b) Buffer concentrations in the range $(0.15-0.25)M$.

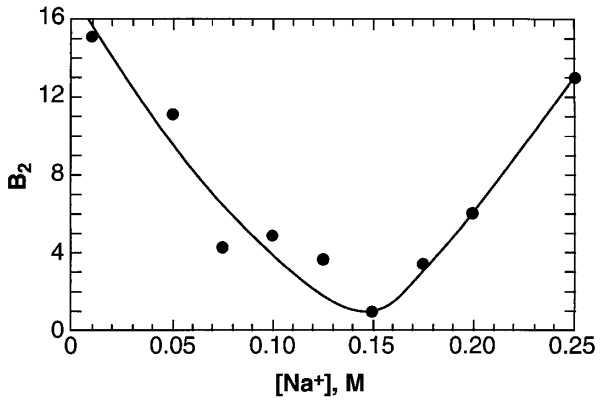

FIG. 2. Dependence of the second osmotic virial coefficient B_2 on the concentration of sodium ions $[Na^+]$, corresponding to Fig. 1. Curves are guides for the eye.

values well within the limits established with other systems. While this correspondence supports the conclusion about the action of these two types of forces, it should not be construed as a critical test of the applicability of concrete forms of the electrostatic or hydration potentials to the studied system.

The dimensionless second virial coefficient depends on the pair interaction potential $U(r)$ (r is the distance between the centers of molecules) as

$$B_2 = 12 \int_0^\infty d\tilde{r}\{1 - \exp[U(\tilde{r})/kT]\}\tilde{r}^2, \qquad \tilde{r} = r/2a, \tag{5}$$

where kT is the thermal energy. The second virial coefficient is an integral quantity and its experimentally determined values cannot yield any insight into the changes of the interaction energy with distance from the molecule.

For charged spheres the electrostatic component in $U(r)$ is [20]

$$U_{\mathrm{el}}(r) = \frac{(z_0 e)}{\varepsilon} \frac{\exp(2\kappa a)}{(1 + \kappa a)^2} \frac{\exp(-\kappa r)}{r}, \tag{6}$$

and, assuming low particle charge, the virial coefficient becomes [21]

$$B_2 = 4 + \frac{3(z_0 e)^2}{2\varepsilon kTa} \frac{(1 + 2\kappa a)}{(1 + \kappa a)^2(\kappa a)^2}. \tag{7}$$

In Eqs. (6) and (7) z_0 is the number of charges per molecule, e is the elementary charge, ε is the dielectric constant of the solvent, κ is the screening parameter defined by $\kappa^2 = (4\pi e^2/\varepsilon kT)_i n_i z_i^2$, n_i and z_i are the number ionic concentration and charge, respectively. The first term in Eq. (7) accounts for "hard spheres" interactions while the second term accounts for the electrostatic contribution.

In agreement with Eq. (7), Fig. 2 shows that the repulsion at low ionic strength decreases with the addition of Na^+: B_2 decreases from $\sim$15 to $\sim$4. Values of B_2 below 4, characteristic of hard spheres potential, could be a manifestation of weak attractive forces (i.e., van der Waals). Assuming that the virial coefficient results below $0.15M$ Na^+ are entirely determined by electrostatic and hard sphere interactions we can find the apoferritin molecular charge z_0 by fitting the experimental points in the range $0.01-0.15M$ with Eq. (7). We get $z_0 = 24$, i.e., the net charge of the apoferritin molecule is negative 24. Apoferritin has 624 acidic and 576 basic amino acid residues on the molecular surface. Dissociation of the acidic groups results in negative charges of the ionized residues, while protonation of the basic groups creates positive charges. Comparing these three numbers, we see that 24 is a feasible molecular charge at used $p\mathrm{H} = 5$, slightly above the isoelectric point of 4.0.

We can now test *a posteriori* if Eqs. (6) and (7), which directly follow from linearized Poisson-Boltzmann equations, are applicable. It has been shown that linearization is a good approximation for values of $e\Psi_s/kT$ (where Ψ_s is the particle surface potential) all the way up to $\lesssim 1$ [22]. In our case, $e\Psi_s/kT = z_0 e^2/[\varepsilon kTa(1 + \kappa a)]$ with $z_0 = 24$, reaches $\sim$0.8 for buffer concentration $0.01M$, and, since κ is higher, is lower than that for higher buffer concentrations. Hence, the "low charge" assumption employed above is valid.

Unfortunately there exists no straightforward relation between salt concentration and hydration energy. Hence, we estimate this energy from the formula derived from fits to experimental data [3,11,23]

$$U_{\mathrm{hyd}}(r) = \pi a L f_0 \exp[-(r - 2a)/L]. \tag{8}$$

Here f_0 and L are empirical parameters for the surface energy density and decay length, respectively. Surface force measurements yield $f_0 \approx 3-30\ \mathrm{mJ/m^2}$ and $L \approx 0.6-1.1$ nm [3,13]. Other studies suggest that the values of L can be even higher [15]. In Fig. 3 we have plotted B_2 as a function of f_0 and L according to Eqs. (5) and (8) and shifting the B_2 values up by 4 to account for the hard spheres repulsion. Choosing decay length $L = 2 \times 0.72$ nm $= 1.44$ nm (twice the diameter of a hydrated sodium ion [3]), we see that the order of magnitude of B_2 is close to the values measured $[Na^+] > 0.15M$. The value $B_2 \approx 13$ measured at $0.25M$ corresponds to $f_0 = 12.5\ \mathrm{mJ/m^2}$, which is in the middle of the range determined by surface force measurements [3,13]. The screening parameter above $0.15M$ buffer concentration is of the order or less than the diameter of hydrated sodium counterion; hence the electrostatic contribution to the virial coefficient has been ignored in the high salt region.

The observed non-DLVO repulsion at high salt concentrations suggests that the small ions are an important factor in the protein intermolecular interactions. Their role is not limited to lowering the solvent dielectric constant and electrostatic screening but may involve interactions with the

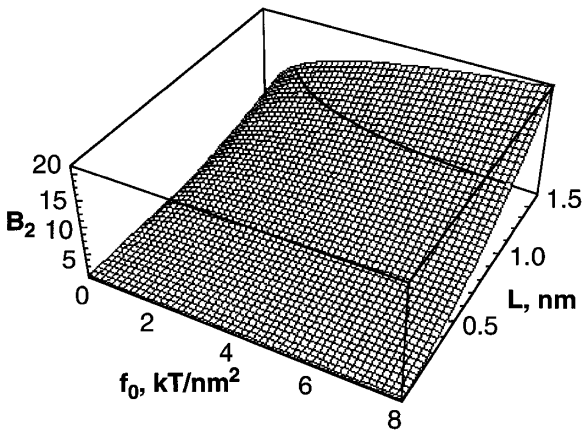

FIG. 3. Calculation of the second osmotic virial coefficient B_2 for hydration and hard spheres interactions as a function of the surface energy f_0 and decay length L using Eqs. (8) and (5), and accounting for hard sphere interactions by adding 4. Solid line corresponds to $B_2 = 13$; see text.

protein molecules that, as in this case, modify the associated solvent structures. In solutions of apoferritin that bear a net negative charge at $p\mathrm{H} = 5.0$, we concluded that the Na^+ counterions are mostly responsible for the effect. In comparison, the protein lysozyme in NaAc buffer at a similar $p\mathrm{H} = 4.7$ displays no evidence for increased repulsion at high ionic strengths [5]. At the studied $p\mathrm{H}$ lysozyme is positively charged and the counterions in this case are the negative acetate ions. These ions are larger and have hydrophilic and hydrophobic ends [24]. The structure and size of the acetate ions affect their hydration ability and, hence, it is not surprising that they do not induce similar effects. The difference between these two proteins may be further amplified by the smaller hydrodynamic radius a of the lysozyme molecules, which, according to Eq. (8), implies weaker hydration interaction.

In summary, we have shown that intermolecular repulsion may be present in protein solutions even at high electrolyte concentrations provided by the buffer, at which electrostatic interactions are suppressed. This explains the stability of proteins at high salt concentrations and the necessity of additives like Cd^{2+} to induce crystallization [7].

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